

Practical use of immobilized lysozyme for the remediation process of *Escherichia coli* in aqueous solution

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The lysozyme enzyme was immobilized on vitreous surface (fragments with diameters of 0.3 and 1.0 mm) for remediation of the microorganism *Escherichia coli* JM 109 into fresh water and saline solutions with 0.9% NaCl (w/v). Characterization of enzymatic film was carried out by infrared spectroscopy and atomic force microscopy techniques. Bactericide activity of the enzyme was evaluated by spectrophotometric analysis. It was verified that the enzymatic film was strongly coupled with the vitreous surface. The topographic analysis demonstrated that the deposited film was uniform and homogeneous. It was observed bactericide activity of film deposited on vitreous surface with 0.3 mm in fresh and saline solutions. This fact was not verified to vitreous fragments with 1.0 mm of diameter.

The constant expansion of different human activities carries into the water certain types of microorganisms that can be incorporated in our alimentary chain. Therefore, mankind should be concerned for microbiologic decontamination standards and looking for actions that be able to limit additional risks to the public health: the chronic effects of contamination of the environment.

The conventional treatments used, nowadays, not eliminate totally the micro contaminants of biological origin. This fact demands that water sources be free from microorganisms or if this is impossible, more appropriate methods of treatment should be applied.

The polymeric films have been classified and proved to be applicable to eliminate, from several origins, contaminated particles from water. Weber-Shirk and Dick (1997) have reported the considerable efficiency in the removal of bacteria, such as, *Escherichia coli*, protozoans and the poliovirus in water by filtration into hydrophilic membranes and superficial films of polymeric structures. Ruggaber and Talley (2006) highlighted the great potential of the enzymatic films for remediation of polluted environments. The advantage of polymeric films are mechanic resistance, the control of thickness and growth rate (Liapis et al. 1989; Marino et al. 2003; Lavigne et al. 2004; Conte et al. 2006; Ruggaber and Talley, 2006).

The fundamental requirement for biological film fixation is that when in solution, the molecules show regions with defined superficial charges for electrostatic interaction. On the whole, the self-assembly technique is very simple due to the proper procedures, equipments and raw materials used in its preparation. The organic layers are obtained by successive immersions in aqueous solutions followed by drying process of these deposited layers. The final properties and the structure of the self-assembly films depend on the experimental parameters (solvents, concentrations, ionic strength, pH etc.), which can affect the final permeability of the membrane (Borato et al. 1997; Knight et al. 2000; Shing-Yi et al. 2000; Miano et al. 2005; Pechkova et al. 2005).

There are different supports for enzymatic immobilization, for example, chitosan and alumina (Sobral et al. 2003). Indubitable, the largest advantage of the use of enzymatic film is that the method used has high capacity to process required chemical reactions.

The lysozyme film has called researches' attention in last years on account of the enzymatic activity being useful in the attack on many bacteria, with the breaking of mucopolysaccharides structure of the cell wall (Masschalck et al. 2001; Datta et al. 1973; Park et al. 2004). Moreover, the lysozyme has an important characteristic, when crystallized, it supports temperatures up to 120°C without perceiving the loss of its enzymatic activity (Kudou et al. 2003; Lavigne et al. 2004).

The lysozyme film acts directly on gram positive bacteria and there is the possibility of interaction with phospholipids of external membrane of gram negative bacteria. It checks the enzyme, thus functions as a protection together with large amounts of biological fluids where the same is found (Masschalck et al. 2001; Min et al. 2005). The specific function of enzyme is represented by a deep opening inside the molecule. Inside this enzyme, the residues Glu-35 and Asp-52 are responsible for the hydrolysis of the glycosidic connection. It is believed that the Glu-35 has an acetylic link to the substrate, while the negative charge of the Asp-52 stabilizes the result of the ion cation (Datta et al. 1973; Dehong and Lu, 2004).

This work had the objective to evaluate the practical use of the immobilized lysozyme enzyme on vitreous surface for remediation process of pathogenic microorganism

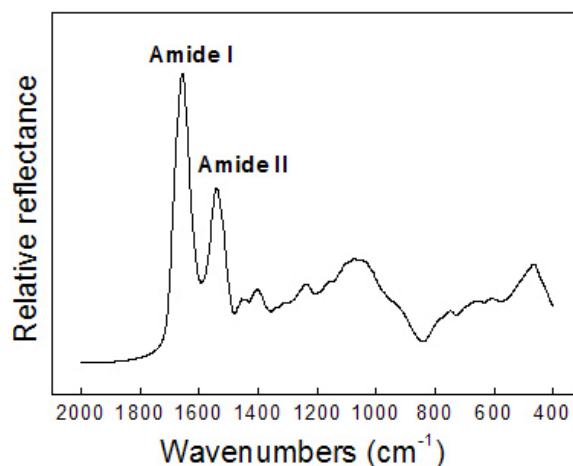


Figure 1. Infrared spectrum for the lysozyme film deposited on vitreous fragments with diameters of 0.3 mm. The tops amide I and amide II correspond to 1542 and 1659 cm^{-1} , respectively.

Escherichia coli JM 109 found in fresh water and saline solution.

MATERIALS AND METHODS

Preparation of the surface and film deposition

The lysozyme film was prepared using the self-assembly technique, which is based on spontaneous irreversible adsorption of the protein. The deposition of the enzyme occurs through covalent chemical bonding between the substrate and the adsorbent (Dickerson and Geis, 1993).

The substrate (fragments of glass of 0.3 and 1.0 mm) was submitted to chemical cleaning and hydrophilization, according to method proposed by Borato et al. (1997). H_2SO_4 (Merck) and H_2O_2 (Merck) solutions were prepared in proportions of (7:3, v/v). Afterwards, the fragments of glass were immersed in this solution at temperature of $80^\circ C$ for 30 min, followed by exhaustive washes in Milli-Q distilled water.

These same surface was submitted to solution of H_2O/H_2O_2 (Merck)/ NH_4OH (Merck) in proportions of (5:1:1, v/v) at temperature of $80^\circ C$. This procedure was carried out for 30 min and later were done various washes in distilled water.

This series of actions generated negative charges onto the glass surface.

The lysozyme aqueous solution from hen egg-white lysozyme (Sigma Chemical, 99%, $47,000 \text{ unit mg}^{-1}$, in the

lyophilic form) was prepared in concentration of $10^{-4} \text{ mol L}^{-1}$ and pH 6.4 under moderate agitation for 4 hrs.

The enzymatic film was immobilized by immersion of the vitreous surface into the lysozyme aqueous solution for 2 hrs under gentle agitation. They were dried under vacuum at room temperature.

The atomic force microscope (TopoMetrix model TNX 2010) and the infrared spectrometer with Fourier analysis (BOMEM model DA8) coupled with MCT detector were utilized to confirm the presence of the film on the vitreous surface.

Preparation of contaminated solution with *Escherichia coli* JM 109

The *Escherichia coli* JM 109 stump was isolated from Eosin Methylene Blue Agar (Merck) and the growth of the colony was made in Trypticase Soy Agar (Merck), at $23^\circ C$, in incubator BOD (Fanem model 347).

The growth of the bacteria occurred in mixture EC Bouillon (Merck). The tubes remained in water bath at temperature of $44.5^\circ C$ for 24 hrs. The development of the colony was detected visually although turbidness and the formation of gas in Dühran tube.

Building of the filtration column

The column, in which the filtration was carried out, was constituted of two separable bodies and united by thread

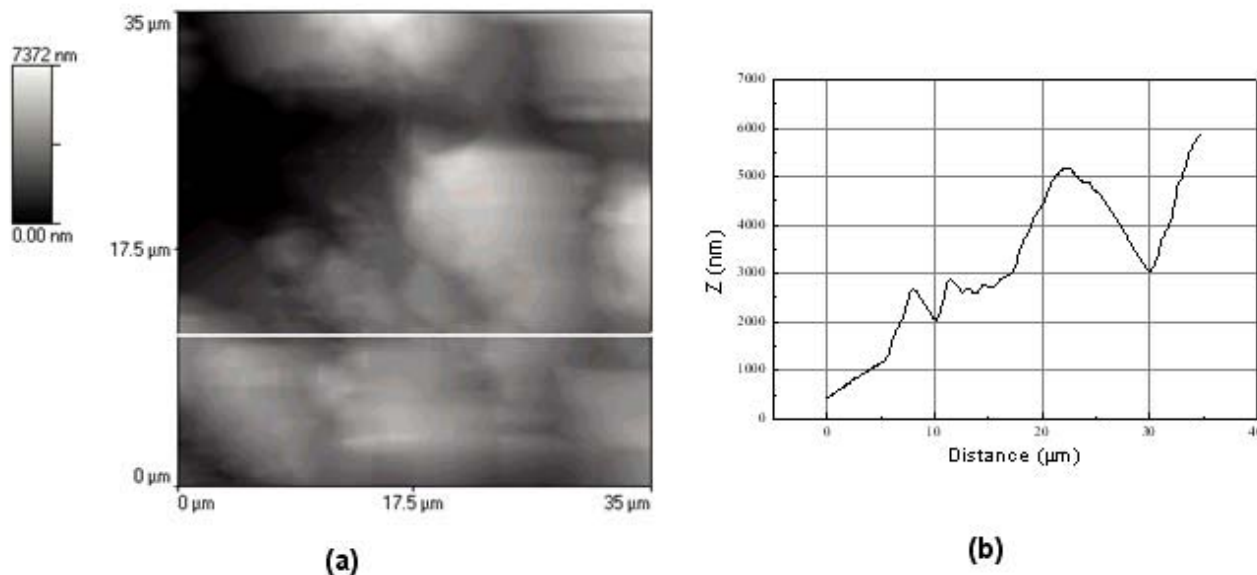


Figure 2. Lysozyme film deposited on vitreous fragments with diameters of 0.3 mm. (a) Topographic Micrograph of the vitreous substrate with diameter of 0.3 mm. (b) Graphic representation of the covered surface with proteic film.

system. The superior part was formed by an acrylic tube of capacity of 50 mL whereas the inferior part was made of glass and had a tap.

Monitoring of the bactericide activity of the enzyme

The substrate of recycled glass with different sizes covered with lysozyme enzyme was packed inside filtration column. The *Escherichia coli* JM 109 solution was diluted in fresh water and salt water with solution of 0.9% NaCl (w/v) and added slowly into column.

The analysis of affluent (solution that drained off from the column) was performed by spectrophotometric measurements (Shimadzu model 1601PC) and the efficiency evaluation followed the Kawabata et al. (1996) relation:

$$\text{Efficiency (\%)} = (\text{PFU}_{\text{inf}} - \text{PFU}_{\text{eff}}) / \text{PFU}_{\text{inf}} \times 100$$

Where PFU_{inf} and PFU_{eff} are the concentration of the specimens in the influent (solution added into column) and affluent suspension, respectively. The tests of bactericide activity were done in triplicate in order to have reliable results.

RESULTS AND DISCUSSION

Presence of immobilized lysozyme on vitreous substrate

After depositing the lysozyme film on vitreous surface (fragments with diameters for values 0.3 and 1.0 mm), measurements of infrared spectrometry were carried out to confirm the deposit formation (Pérez and Griebenow, 2000) and the topographic analysis were done to verify its distribution.

The Figure 1 illustrates the infrared spectrum ranging between 400 to 2000 cm^{-1} for lysozyme film on vitreous fragments with diameters of 0.3 mm. The bands ranging between 1237 and 1401 cm^{-1} are related to amino acid tryptophane; another characteristic of the lysozyme is also observed in the region between 1530 and 1700 cm^{-1} (bands in 1542 and 1659 cm^{-1}), which corresponds to the amino band, as verified in the literature (Alix et al. 1985). The bands were similar for the two granulometries confirming the presence of lysozyme on them.

Figure 2a is a topographic image of small part of the vitreous substrate with diameter of 0.3 mm covered with the enzymatic film, made up of approximately 30 overlapping layers (Borato et al. 1997).

Figure 2b is a graphic representation of a part of the covered surface with proteic film. It is noted that the vitreous surface is covered homogeneously by enzymatic film. The topographic analysis in micrometric scale not revealed difference between the two granulometries.

Bactericide activity of protein film

Previous experiments showed promising results for bactericide activity of immobilized lysozyme on vitreous substrates with diameters equal or inferior to 0.3 mm. However, size of fragments smaller than 0.3 mm have as disadvantage to provide slow speed of liquid by column. In this work, we showed glass fragments with granulometries of 1.0 mm to compare size bigger than 0.3 mm.

We also tested high concentration of microorganisms and dissolved salt in water to evaluate if in extreme conditions the enzymatic activity was remained. In comparative experiments prepared with fresh water and salt-water solutions, it was noticed that the enzyme maintains its bactericide activity for concentrated solutions with approximately 6,000 bacteria/ cm^3 .

We used the spectrophotometric technique because this is one of the simplest ways to determine the density of a bacterial culture. In dense culture, the light is scattered, and less light reaches the phototube of the spectrophotometer. In fact, turbidity is more closely related to cell biomass (cell dry weight) than to the number of cells present. In dilute samples, absorbance is directly proportional to biomass.

In the presence of the immobilized lysozyme occurs the rupture cell of the bacteria. This fact is evidenced with a significant decrease in the optical density of wavelength 600 nm (visible spectrum). We verified that in the presence of lysozyme the optical density showed a progressive decrease of the turbidity, resultant from effect of the bactericide activity. Moreover, it was observed that this activity has shown reduction of the turbidness on the first 30 mL, confirming the process of lysis cell as presented in Figure 3. After sieving 30 mL of contaminated solution in column, it was noted decreases in remediation process, probably owing to the fact that the film is covered with

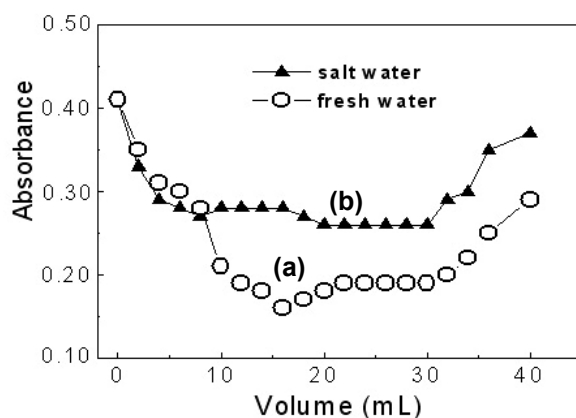


Figure 3. Bactericide activity of the lysozyme deposited on vitreous granular surface of 0.3 mm, for *Escherichia coli* in fresh water and salt water.

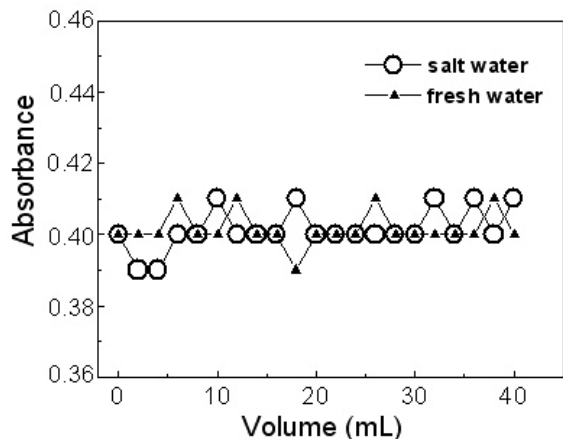


Figure 4. Action of lysozyme film deposited on vitreous granular surface of 1.0 mm against bacteria in fresh water and salt water.

bacterial fragments, thereby, hindering the effective action of the enzyme.

Figure 3 (curve a) indicates that the bactericide efficiency of lysozyme in fresh water was on average 60%, this results are near to 50% found for Assis and Claro (2003). On the other hand, in curve b, for the saline solution, the efficiency was on average 30%. It was observed that the activity of the immobilized enzyme is reduced because of the presence of ions (electrolytic solution of NaCl). According to Campbell and Dwek (1984), when ions are present, the lysozyme in solution undergoes a modification in its conformation. In this way, there is a great loss of the enzymatic activity.

The influence of the glass particles with immobilized lysozyme in the remediation efficiency, it was also tested on vitreous surface with diameter of 1.0 mm. In Figure 4, it is no observed variation in the absorbance for solutions of bacteria, indicating that there is no bactericide activity of the lysozyme. The glass fragments with diameter of 1.0 mm leave big spaces for bacterial solution to drain off by column. This fact becomes ineffective the percolation process because the time of contact between immobilized protein and *Escherichia coli* is very short, thus the solution of microorganism passes by enzymatic film without undergoing bactericide action of the lysozyme.

In this work, we demonstrated that the lysozyme efficiency in the remediation process depend on size substrate and other factors that can affect the enzyme and consequently the specific action sites, as was seen in saline medium.

CONCLUDING REMARKS

Lysozyme enzymatic film grew on recycled glass fragments of 0.3 mm has potential for applications in decontamination systems, especially in no saline medium,

in function of the good bactericide activity and the low cost. Contra wise, the enzymatic film deposited on fragments of 1.0 mm had not bactericide action in solutions with approximately 6,000 bacteria/cm³. One of the biggest problems for the practice application of methodology developed (decontamination system) is in the fact of fragments of bacteria's cell wall to accumulate on the enzymatic film obstructing the lysis of other bacteria.

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